PCT/EP03/02553

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Rec'd PCT/PTO 13 SEP 2004

REC'D 08 JUL 2003
WIPO PCT

Kongeriget Danmark

Patent application No.: PA 2002 00381

Date of filing: 13. March 2002

Applicant:
(Name and address)
Nordic Bioscience Diagnostics A/S
Herlev Hovedgade 207
2730 Herlev
Denmark

Title: Detection of Specific Nitrated Markers.

IPC: -

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Patent- og Varemærkestyrelsen
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Patent- og Varemærkestyrelsen
Økonomi- og Erhvervsministeriet

11. June 2003

John Nielsen

PATENT- OG VAREMÆRKESTYRELSEN

Detection of Specific Nitrated Markers

Technical field of the invention

The present invention relates to a method for monitoring oxidative damage especially in relation to an inflammatory condition. Detection of nitrated specific proteins or fragments thereof versus equivalent non-nitrated proteins or fragments thereof, may serve as an index for oxidative damage in for example inflammatory bowel disease, systemic lupus erythematosus, arthritis, cancer, Parkinson's or Alzheimer's disease.

Background of the invention

Throughout a lifetime organisms are challenged with numerous events and conditions that generate reactive oxygen species (ROS). An increase in an organisms rate of ROS production or a decrease in their rate of scavenging will increase the oxidative modification of cellular molecules, including DNA and proteins. Oxidation may have deleterious effects on protein function and stability. Many enzymes have been shown to loose their biological activity as a consequence of oxidation. Other effects of oxidation are lowered temperature-stability and changes in protein susceptibility towards proteolysis; the later might lead to an accumulation of oxidized proteins unable to undergo degradation. Protein oxidation may be implicated in the pathogenesis of several diseases such as neurodegenerative diseases, cancers, arteriosclerosis, cataractogenisis, dysplasia, dystrophia and inflammatory diseases as well as in normal ageing.

Some of the common ROS generating processes and systems known to modify proteins are irradiation, inflammation, metal catalysed reactions such as Fe(II) or Cu(I) reduction and various other oxidizing compounds or free radicals, including nitric oxide (NO), peroxynitrite, H_2O_2 or hydroxyl, hydroperoxyl superoxide and lipid peroxyl radicals. A number of ROS are formed by specific

enzyme systems such as nitric oxide synthetase (NOS), cyclo-oxygenase and mono-amine oxidase B, whereof some are induced under inflammatory conditions.

Under normal conditions the oxidative potential in the micro-environment of an organism is under tight control by a number of balancing systems including antioxidants, free radical scavengers, reductases, peroxidases, catalase, glutathione-S-transferase, superoxide dismutase and various metal-binding proteins. These systems can be viewed as protection mechanisms, more than repair systems. Actual repair mechanisms specific for oxidative damaged proteins are rare, whereas the oxidative damage to nucleic acids is subject to highly efficient repair systems (Stadtman and Levine 2000).

Nitric oxide (NO) is produced in many tissues and regulates diverse functions, such as smooth muscle relaxation, non-specific defence against microorganisms, neurotransmitter and a possible modulator of the cartilage matrix. Nitric oxide synthetase (NOS) is responsible for the production of NO. There are two classes of NOS, a constitutive (cNOS) and an inducible (iNOS) form. iNOS activity appears in response to various cytokines, and produces a much larger amount of NO than cNOS. iNOS activity is thought to account for the proinflammatory effects of NO, as seen in conditions such as inflammatory bowel diseases, spontaneous gut inflammation, cardiovascular inflammation and arthritic diseases (osteoarthritis (OA) or rheumatoid arthritis (RA)).

The large cytotoxicity of NO is partly due to its ability to react with superoxide anion (O_2^-) to generate peroxynitrite anion ($ONOO^-$) and its conjugate acid, peroxynitrous acid (ONOOH). At neutral pH $ONOO^-$ is partly protonated, generating ONOOH, which rapidly decomposes to nitrate. These strong oxidants might seriously compromise cellular regulation, as it is capable of nitrating aromatic compounds like free phenylalanine, tyrosine and tryptophan as well as peptide chains containing these amino acids. This result in nitrophenylalanine, nitrotryptophan and nitrotyrosine, the later can also be generated through the combined hydroxylation and nitration of a phenylalanine residue (Lin et al 2000). The nitration is irreversible

and inhibits the phosphorylation of tyrosine and tryptophan residues, thus interfering with signal transduction pathways.

In OA and RA, NO is produced in large amounts by chondrocytes, macrophages and inflamed synovium. A high level of nitrite/ nitrate has been found in the synovial fluid, serum and urine of patients with OA and RA (Lotz 1999). However, elevated NO levels cannot be considered a specific marker for any given disease or condition, as several different processes and tissues can give rise to systemic elevated NO levels.

The major clinical manifestation of RA as well as OA is an abnormal and degraded cartilage However, until now it has been difficult to directly assess the ongoing cartilage destruction in arthritis patients, because specific markers for this process have not been available in the clinical practice. At clinical diagnosis of OA and RA, damage to cartilage in joints is recorded by X-ray, which reveals a loss of joint space as cartilage is destroyed and lost. Furthermore the patients are scored according to the pain and mobility problems caused by the joint destruction, but even though a number of standardised rating systems have been introduced, it is difficult to quantify these parameters. Other markers used for assessment of RA patients, such as C-reactive protein and Rheumatoid factors are associated with the inflammatory process involved in the disease, but are probably not directly related to the level of cartilage destruction and they are not specific for RA.

Detection of metabolites, such as cartilage oligomeric matrix protein (COMP), hyaluronates, aggrecan and collagen type II or III fragments arising from destruction of joints affected by inflammatory disease have been reported (Moller 1998, Wollheim 1996, US patent 5919634, US patent 6132976 and PCT application WO 01/38872). The clinical usefulness of these markers, however, remains to be proven.

The detection of NO_2-modified amino acids is known from the PCT patent applications WO 96/04311 and WO 98/29452. These patent applications disclose the sequence independent detection of a nitrotyrosine or a nitrotryptophan residue in a

protein or in its free form using an antibody, which specifically recognizes the nitro-group. Such an antibody might be used to assess a pathological condition relating to an abnormal level of nitrotyrosine. However the antibody will not be able to assess the problem in relation to a specific tissue or protein as it recognizes nitrotyrosine independent of the surrounding amino acid sequence.

The present invention relates to methods for quantifying NO_2-modified amino acids within the specific context of a protein or fragments thereof. Determination of such nitrated specific proteins or fragments thereof enables an assessment of oxidative damage and metabolic state of the given protein. This will enable the generation of diagnostic assays, which associate metabolic changes and oxidative damage in specific diseases.

Summary of the Invention

According to the present invention a method for detecting oxidative damage in a mammal is provided. The method enables such monitoring by detecting one or more nitrated aromatic residues in combination with a specific amino acid sequence of a protein or peptide, preferably specific to a certain tissue, in a biological sample. Preferred aromatic amino acid residues are nitrotyrosine and/ or nitrotryptophan.

The method of the present invention can be applied for monitoring a pathological process involving an oxidative damage correlating with non-inflammatory diseases like arteriosclerosis, cancer, Alzheimer's disease, Parkinson's disease or inflammatory diseases like asthma, cardiovascular inflammation, diabetes, inflammatory bowel disease, psoriasis, systemic lupus erythematosus, arthritis.

The method of the present invention will enable the monitoring of a catabolic process of a joint tissue by measuring a protein or peptide derived from the extra cellular matrix of cartilage, joint synovium or subchondral bone. Such a protein might be collagen types I, II, III, VI, IX or XI, aggrecan, biglycan, chondromodulin,

cartilage link protein, cartilage oligomeric matrix protein, cartilage intermediate layer protein or a fragment thereof, wherein a nitrotyrosine and/ or nitrotryptophan residue is located.

The detection performed in the method of the present invention is carried out using an antibody, which specifically binds a nitrated epitope, comprising at least one nitrated aromatic amino acid residue in conjunction with a specific amino acid sequence.

The detection performed in the method of the present invention can also be carried out utilizing two antibodies, one which is specific for the nitrated aromatic amino acid residue and one which recognizes the specific amino acid sequence.

The detections of the present invention can be performed in a way that generate an index of oxidative damage/ inflammation, by using a second antibody, which specifically binds an equivalent non-nitrated amino acid sequence as the antibody which recognizes the nitrated epitope described above, thereby generating an ratio between a specific nitrated and non-nitrated protein or peptide.

The index of oxidative damage/ inflammation generated by application of the present invention, to a sample from a mammal, can be used to provide means for diagnosis or assessment of the severity of a disease involving oxidative damage, especially joint tissue diseases. For these purposes a kit utilizing an antibody, which recognizes a nitrated epitope and a second antibody recognizing the similar non-nitrated epitope, together with suitable labels, is provided. A supplement to such a kit is a peptide, which contains a succession of amino acids equivalent to the binding epitope for one of the mentioned antibodies, for competition assays. The kits of the present invention can be applied to samples like mammalian body fluids, extracts from cells or tissues or supernatants from cells or tissues cultured in vitro.

The present invention especially relates to nitrated collagen type II protein, wherein the nitrated amino acid is the tyrosine of one of the sequences His-Arg-Gly-Tyr-Pro-Gly-Leu-Asp-Gly or Leu-Gln-Tyr-Met-Arg-Ala. Synthetic nitrated peptides including these sequences are used to raise polyclonal and/ or monoclonal antibodies, as well as cell lines producing such monoclonal antibodies.

Detailed description of the Invention

The present invention is based upon a new approach for development of diagnostic and prognostic assays for monitoring pathological conditions in of mammalian tissues, combining metabolic tissue specific markers with the state of an oxidative/ inflammatory condition. The invention relates to the fact that one of the key effects of oxidative damage is nitration of aromatic amino acid residues such as phenylalanine, tyrosine or tryptophan. The presence of one or more nitrated aromatic amino acid residues in or near a marker specific to a certain tissue or disease, provides information about the metabolic state of a tissue from which a marker originates as well as the oxidative condition of the same tissue.

As used herein, "antibody" means polyclonal, monoclonal or humanized antibodies, including Fc fragments, Fab fragments, chimeric antibodies or other antigen-specific antibody fragments.

As used herein "biochemical marker" or just "marker", means a protein, protein fragment, polypeptide, domain structure, peptide or otherwise proteolytical processed protein, representing changes within a specific tissue, which becomes detectable in relation to such changes.

As used herein, "nitrated epitope", means a site within an antigen containing a nitrophenylalanine, nitrotyrosine or nitrotryptophan residue, where the epitope recognized by an antibody constitutes the nitrated residue and enough adjacent amino acid residues to gain protein specificity of the antibody.

As used herein, "two independent epitopes", means two sites within the same protein, polypeptide or peptide recognized by two different antibodies. Preferably one of the sites is a single nitrated aromatic amino acid residue

As used herein, "nitrated aromatic amino acid residue", means a phenylalanine, tyrosine or tryptophan residue situated in a protein or peptide, where the aromatic ring of the amino acid residue has been modified by covalent attachment of a NO_2-group.

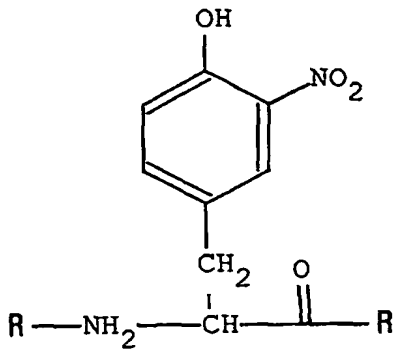
As used herein, "nitrophenylalanine" means a phenylalanine residue situated in a protein or peptide, where the aromatic ring of the phenylalanine residue has been modified by covalent attachment of a NO_2-group.

As used herein, "nitrotryptophan", means a tryptophan residue situated in a protein or peptide, where the aromatic ring of the tryptophan residue has been modified by covalent attachment of a NO_2-group.

As used herein, "nitrotyrosine", means a tyrosine or phenylanaline residue situated in a protein or peptide, where the aromatic ring of the tyrosine has been modified by covalent attachment of a NO_2-group or the aromatic ring of the phenylalanine residue has been modified by combined covalent attachment of an OH-group at position 4 and a NO_2-group in one of the remaining positions.

As used herein, "Tyr:NO_2", means nitrotyrosine. The preferred position of the nitro-group is adjacent to the hydroxyl-group, for example as shown in Formula I.

Formula I



In one embodiment of the present invention a protein, peptide or fragment thereof containing a nitrated aromatic amino acid residue is detected by means of identification, in which the identification relies on detection of the nitrated aromatic residue, in combination with a specific amino acid sequence. The nitration of the aromatic amino acid has arisen as a result of oxidative damage, preferably connected to an inflammatory condition.

In one preferred embodiment of the present invention the nitrated aromatic amino acids to be measured are nitrotyrosine and/ or nitrotryptophan residues. Most preferred are nitrotyrosine residues, which can either be generated through the nitration of a tyrosine residue, or through the combined hydroxylation and nitration of a phenylalanine residue.

In pathological processes involving oxidative damage, the degree of nitrated proteins can be used to assess the severity of this damage.

In one embodiment of the present invention this is assessed by generating an index of oxidative damage by measuring a nitrated residue in conjunction with its surrounding sequence of a protein, peptide or fragment thereof containing a nitrated aromatic amino acid residue versus the equivalent non-nitrated protein, peptide or fragment thereof, with the exception that a nitrotyrosine may be a phenylalanine in the non-nitrated peptide.

Another embodiment of the present invention includes its application for in-vitro diagnosis or assessment of severity of a disease connected to an oxidative pathology and/ or inflammatory condition. Such diseases could be, but is not limited to, arteriosclerosis, Alzheimer's, asthma, Chrons disease, Parkinson's disease, cancer, cataractogenisis, diabetes, bronchopulmonary dysplasia, multiple sclerosis, muscular dystrophy, inflammatory bowel diseases, psoriasis, systemic lupus erythematosus, osteoarthritis or rheumatoid arthritis

Inflammation of joint tissues is seen in conjunction with arthritic diseases such as RA and OA or as a result of an acute joint injury. This inflammatory condition is very often connected to a catabolic process within the tissue, leading to its gradual degradation. Such catabolic processes within a tissue, releases proteins, peptides or fragments thereof to numerous body fluids, like the synovial fluid, blood, serum or urine. Such molecules can be utilized as biochemical marker(s) for joint tissue degradation/ catabolism. Protein or protein fragments derived from the cartilage matrix or subchondral bone, containing at least one tyrosine, phenylalanine or tryptophan residue are more likely to become nitrated upon the inflammatory increase in NO production as seen in OA and RA. Measuring nitrated versus non-nitrated catabolic markers enables the correlation between degradation and inflammation. This principle can also be applied to markers for gut inflammatory diseases or other diseases where oxidative NO_2-modifications occur, such as in arteriosclerosis, cancer, Alzheimer's or Parkinson's disease.

In one embodiment of the present invention the nitrated aromatic residues arisen as a result of an inflammatory condition in a joint tissue is situated in a protein, peptide or fragment thereof derived from the extra cellular matrix or cartilage, joint synovium or subchondral bone, which can be measured in order to monitor the catabolic process in the tissue.

In one preferred embodiment, the protein or fragment thereof, which is monitored, constitutes a marker of cartilage degradation associated with an

inflammatory joint disease The nitrated form versus the non-nitrated form of such a cartilage matrix derived marker is measured generating an index of inflammation.

Proteins or fragments thereof, which can be nitrated and act as markers of interest include, but is not limited to, collagen types I, II, III, VI, IX or XI, aggrecan, cartilage link protein, cartilage oligomeric matrix protein, cartilage intermediate layer protein.

A preferred marker protein is collagen type II. Collagen type II contains two tyrosine's, which can be nitrated upon oxidative damage. The first sequence His-Arg-Gly-Tyr-Pro-Gly-Leu-Asp-Gly is localised in the triple helical region while the second sequence Leu-Gln-Tyr-Met-Arg-Ala is located in the non-helical domain at the C-telopeptide. The amino acids sequences including the tyrosine residues are specific for type II collagen and can be employed as specific biochemical markers of catabolic processes in the cartilage tissue.

A preferred embodiment is the detection of the nitrated type II collagen marker versus the equivalent non-nitrated type II collagen marker providing an index of the oxidative damage and/ or the inflammatory condition of the cartilage tissue in associated with the metabolic condition of the same tissue. The principle of this method also applies to other tissue specific markers originating from oxidative damaged tissues undergoing metabolic changes at the same time.

Especially for monitoring the results of a treatment this is of importance, as some forms of treatment might influence the catabolic process whereas others might influence the inflammatory state. This more differentiated assessment of the disease obtained by application of the present invention will enable therapeutic interventions to be targeted to individual patients.

The detection of a nitrated aromatic amino acid residue in combination with a sequence located within a specific protein, peptide or fragment thereof can be performed in numerous ways, such as, but not limited to, HPLC, mass spectroscopy, iso-electric focusing, sequencing or immunoassays.

One preferred method of detection is the use of an immunoassay, utilizing an antibody, which specifically binds at least one nitrated aromatic amino acid residue

in conjunction with the surrounding amino acid sequence of a specific protein (nitrated epitope). Assay forms in which such an antibody can be applied include, but not limited to, ELISA, microarray, RIA, FACS, Western blotting, immunoaffinity chromatography, and immunohistochemistry.

Another method of detection is a sandwich immunoassay, utilizing an antibody, which specifically recognizes a nitrated aromatic amino acid residue independent of the surrounding sequence and a second antibody, which recognizes a specific amino acid sequence located within the same protein, peptide or a fragment thereof as the nitrated amino acid residue. The second antibody can very well be a polyclonal antibody with specificity towards a specific protein e.g. collagen type II, where the actual epitope has not been identified.

The most preferred method for monitoring a pathological process involving oxidative damage and/ or an inflammatory condition in association with metabolic changes utilizes the generation of an index as described above. More specifically such an index is generated by contacting an antibody, which specifically binds a nitrated epitope, with a biological sample. Upon reaction with the first antibody, the antibody-peptide conjugates can be detected by different affinity/ visualization or isolation methods. If appropriate, a second antibody, which specifically binds an equivalent non-nitosylated amino acid sequence as the first antibody, is contacted with an aliquot of the same biological sample as the first antibody, or the remnants (supernatant) resulting from an isolation of the first antibody-peptide conjugate. The ratio between nitrated and non-nitrated peptide and/ or protein in the biological sample is determined. Methods of determination are well known in the art, for example ELISA, microarray, RIA, FACS, immunoaffinity chromatography, and immunohistochemistry. If applying other methods than chromatography, it is important that the two antibodies are labelled in a manner that enables differentiation between them. This could be different fluoresce (e.g. red, green, yellow), enzymatic label vs. radioactive label and so forth. An index of oxidative damage and/ or inflammation in association with the metabolic condition of the tissue from where

the specific protein originates can be provided by evaluating ratios from a patient in relation to ratios from healthy individuals.

In situations where a tissue sample is used for monitoring of pathological processes in joint tissue, there is a strong likelihood that denatured helical collagen domains, resulting from catabolic processes within the tissue, might be retained in the tissue by cross-linking and fibrillar packaging. To address this problem, the biological sample is first contacted with an enzyme having the ability to selectively cleave unwound (non-helical) collagens without cleaving the His-Arg-Gly-Tyr-Pro-Gly-Leu-Asp-Gly and/ or the Leu-Gln-Tyr-Met-Arg-Ala epitope. Such enzymes could be, but is not limited to, trypsin or chymotrypsin, which are unable to cleave wound (native) collagen within the α-helix. The fragments of unwound collagen are then extracted from the biological sample to produce an extract of unwound collagen fragments. This extract can then be assayed as mentioned in the above.

Antibodies with the properties described above are raised against a peptide constituting a nitrated epitope. The peptide is used as an antigen for immunisation. The peptide is emulsified in an adjuvant medium, preferably incomplete Freund's adjuvant and injected subcutaneously or into the peritoneal cavity of a mammalian host, preferably a rodent most preferred rabbits, even more preferred BalbC mice. To enhance immunogenic properties of the antigenic peptide it can be coupled to a carrier protein before emulsified in an adjuvant medium. Useful carriers are proteins such as keyhole limpet hemocyanin (KLH), edestin, thyroglobuloin, albumins, such as bovine or human serum albumin (BSA or HSA), tetanus toxoid, and cholera toxoid, polyaminoacids, such as poly-(D-lysine-D-glutamic acid). Booster injections may be given at regular intervals until an immune response is obtained, the last injection may be given intravenously to ensure maximal B-cell stimulation.

Antisera will be screened for their ability to bind the desired epitope and their amount of cross reactivity to the non-nitrated epitope. Antisera from the most promising hosts may be used in their crude form or purified.

Monoclonal antibodies may be prepared from the immunised mice with the highest antibody titre, by fusing lymphocytes isolated from the spleen of these mice

with a myeloma cell line. The generated hybridoma clones are screened for their ability to produce antibodies, which recognize the desired epitope. Cell lines can be established for production and purification of monoclonal antibodies.

Methods for polyclonal and monoclonal antibody production are well known in the art and other methods than the described can also be utilized.

In one aspect of the present invention the synthetic peptide for antibody and cell line generation as described above is $(X_{aa})_m$-His-Arg-Gly-Tyr:NO_2-Pro-Gly-$(X_{aa})_n$, wherein X_{aa} denote any amino acid or derivates thereof and m and n are independent integers from 0 to 10.

In one preferred embodiment of the present invention the synthetic peptide for antibody and cell line generation as described above is $(X_{aa})_m$-His-Arg-Gly-Tyr:NO_2-Pro-Gly-Leu-Asp-Gly-$(X_{aa})_n$, wherein X_{aa} denote any amino acid or derivates thereof and m and n are independent integers from 0 to 10.

In another preferred embodiment the synthetic peptides for antibody and cell line generation as described above has the form $(X_{aa})_m$-Leu-Gln-Tyr:NO_2-Met-Arg-Ala-$(X_{aa})_n$, wherein X_{aa} denote any amino acid or derivates thereof and m and n are independent integers from 0 to 10.

With the exception that a nitrotyrosine may be derived from a phenylalanine in the non-nitrated peptide, the second antibodies utilized in the present invention are generated using the same or similar techniques as for the preparation of the nitrosyl binding antibodies.

One embodiment of the present invention constitutes the development of a diagnostic kit for use in detection and monitoring of oxidative damage and/ or an inflammatory condition. This includes an antibody recognizing a nitrated epitope, preferably utilizing an antibody of the present invention, either alone or in combination with a second antibody with specificity towards the equivalent non-nitrated sequence, enabling a simultaneous assessment of tissue metabolism and oxidative damage. The kit can be applied on mammalian body fluids or extracts of cells or tissues, preferably derived from humans. For competition detections a peptide of 6 to 20 amino acids, in which a succession of amino acids is equivalent to

the binding epitope for one of said antibodies, might be supplied either in a labelled or non labelled form. The antibodies may be labelled by joining them, either covalently or non-covalently, with a reporter molecule. Suitable reporter molecules or labels, which may be used for ease of detection, include radioisotopes, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles, and the like. One of the non-labelled antibodies or a peptide of the kit might be immobilised, preferably on a solid surface like a micro-titter plate, possibly by conjugation to a suitable protein carrier like BSA.

In a preferred embodiment the first antibody in the kit described above recognize the nitrosylated collagen type II sequences previously described, and the second antibody recognizes the equivalent non-nitrated sequence.

Brief description of the figures

Figure 1: shows a standard curve for nitrated collagen type II immunoassay in a semi-logarithmic plot. The concentration of free antigen is in nM. B/Bo represents the ratio between antibody bound to coated antigen in the presence of free antigen (B) or in the absence of free antigen (Bo) and is given in percentage.

Figure 2: Shows competitive inhibition of antiserum D37 binding to His-Arg-Gly-Tyr:NO2-Pro-Gly-Leu-Asp-Gly coated plates using His-Arg-Gly-Tyr:NO2-Pro-Gly-Leu-Asp-Gly(.), His-Arg-Gly-Tyr-Pro-Gly-Leu-Asp-Gly(●), native type II collagen (◆), nitrated type II collagen (.), type I collagen (▲), BSA (▲) and nitrated BSA (.) as competitors. B/Bo represents the ratio between antibody bound to coated antigen in the presence of competitor antigen (B) or in the absence of competitor antigen (Bo) and is given in percentage.

Examples

Example 1: Collagen type II immunoassay

Antisera:

A sequence of nine amino acids (His-Arg-Gly-Tyr:NO_2-Pro-Gly-Leu-Asp-Gly) derived from the triple helical region of type II collagen [(α1) II] and a second sequence of six amino acids Leu-Gln-Tyr:NO_2-Met-Arg-Ala derived from the C-telopeptide of type II collagen were synthesized using standard Fmoc solid-phase peptide synthesis (HBTU/HOBt protocol) (Chan,W.C. and White, P.D., 2000).

The amino acids sequence was conjugated to thyroglobulin by a carbodiimide procedure (Soinila et al 1992).

Rabbits were injected intraperitoneally with 1 ml of the conjugate emulsified in complete Freund's adjuvant. The conjugate and the adjuvant were mixed in equal volumes. Injections were repeated four times every month with a similar amount of conjugate in incomplete Freund's adjuvant. Ten days after the last injection, the rabbits were sacrificed for the final bleeding. Blood were collected and centrifuged for 10 minutes at 1500 x *g* at 4°C. The supernatants were stored at –20°C.

The following examples will concentrate on antisera achieved from immunisation with the His-Arg-Gly-Tyr:NO_2-Pro-Gly-Leu-Asp-Gly peptide. All examples can be performed in similar ways for the Leu-Gln-Tyr:NO_2-Met-Arg-Ala peptide.

Six antisera, identified as Coll2-1:NO2 D35, D36, D37, D38 D39 and D40, were obtained and their specificity were tested with the competitive inhibitions His-Arg-Gly-Tyr(NO_2)-Pro-Gly-Leu-Asp-Gly, His-Arg-Gly-Tyr-Pro-Gly-Leu-Asp-Gly, type II nitrated collagen, native type II collagen, type I nitrated collagen I, type I collagen, nitrated BSA and BSA.

Competitive ELISA:

A competitive immunoassay was developed to quantify breakdown products of nitrated type II collagen containing following sequence His-Arg-Gly-Tyr:NO_2-Pro-Gly-Leu-Asp-Gly. Synthetic His-Arg-Gly-Tyr:NO_2-Pro-Gly-Leu-Asp-Gly

peptides were biotinylated and incubated at 1.25 ng/ml on streptavidie coated plates (Nunc, Denmark) for 1 hour at room temperature. Fifty µl of calibrators (to generate a standard curve) or unknown samples, diluted in Ultroser G (Gibco) were added to separate wells. Hundred µl antiserum (see above) diluted 1/125000 was added to each well. Samples were mixed by rotating the plate and incubated 1 hour at room temperature. After three successive washings with washing buffer (Tris 25 mM, NaCl 50mM pH 7.3), 100 µl of horseradish peroxidase-conjugated goat antibodies to rabbit IgG (Biosource, Belgium) were added to each well and incubated 1 hour at room temperature. After washing, 100 µl of freshly prepared enzyme substrate (TMB, Biosource, Belgium) were added to each well. After 15 minutes incubation, the reaction was stopped with 100 µl 4M H_3PO_4. The coloration was read with a microplate reader (Labsystem iEMS Reader MF, Finland) at 450 nm and corrected for absorbance at 620 nm. A standard curve was constructed on a log-linear graph by plotting the B/Bo of 6 calibrators (10 to 0.01 nM) (figure 1). The concentration of HIS-ARG-GLY-TYR:NO2-PRO-GLY-LEU-ASP-GLYcontaining peptides in the samples, were determined by interpolation on the calibration curve.

Example 2: Characterisation of antisera Coll2-1:NO2 D37-40

Specificity

The antisera produced, were tested for their specificity for His-Arg-Gly-Tyr:NO2-Pro-Gly-Leu-Asp-Gly, by use of the immunoassay described in example 1. To test for specificity His-Arg-Gly-Tyr:NO2-Pro-Gly-Leu-Asp-Gly, His-Arg-Gly-Tyr-Pro-Gly-Leu-Asp-Gly peptide, type II nitrated collagen, native type II collagen, type I nitrated collagen I, type I collagen, nitrated BSA and BSA.

Native type II collagen, type I collagen, nitrated collagen type I, nitrated BSA and BSA, was not able to compete with the coated His-Arg-Gly-Tyr:NO2-Pro-Gly-Leu-Asp-Gly peptide in the applied concentrations, whereas the antiserum showed week affinity to the non-Nitrated His-Arg-Gly-Tyr-Pro-Gly-Leu-Asp-Gly sequence and nitrated collagen type II and strong affinity to the His-Arg-Gly-Tyr:NO2-Pro-Gly-Leu-Asp-Gly sequence. Shown for Coll2-1:NO2 D37 in figure 3.

Detection limit

The detection limit of the assay described in example 1, is calculated as the mean (M) Bo value of 21 determinations of standard A minus 3 times the standard derivation (SD) of Bo (M_A-3*SD_A). For Coll2-1:NO2 D37 the detection limit was 25 pM.

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Claims

1. A method for detecting and/ or monitoring oxidative damage in a mammalian protein, peptide or a fragment thereof, comprising detecting one or more aromatic amino acid residues in a nitrated form, in combination with a specific amino acid sequence.

2. A method according to claim 1, wherein the nitrated aromatic amino acid residue(s) and the specific amino acid sequence constitute two independent epitopes.

3. A method according to claim 1, wherein the nitrated aromatic amino acid residue(s) constitutes a single epitope together with the surrounding amino acid sequence.

4. A method according to any of the claims 1 or 3, wherein the nitrated aromatic amino acid residue(s) is located within a biochemical marker.

5. A method according to claim 4, wherein the nitrated aromatic amino acid residue(s) is located in a protein or fragment thereof derived form joint tissue.

6. A method according to any of the claims 1 to 5, wherein the nitrated aromatic amino acid residue(s) is a nitrotyrosine and/ or a nitrotryptophan residue.

7. A method according to any of the claims 1 to 6, wherein the nitrated aromatic amino acid residue(s) is located in collagen types I, II, III, VI, IX or XI, aggrecan, cartilage link protein, cartilage oligomeric matrix protein, cartilage intermediate layer protein or in fragments of said proteins.

8. A method according to claim 7, wherein the nitrated aromatic amino acid residue(s) is located in collagen type II or a fragment thereof.

9. A method according to claim 8, wherein the tyrosine of the collagen type II derived sequence His-Arg-Gly-Tyr-Pro-Gly-Leu-Asp-Gly, is nitrated.

10. A method according to claim 8, wherein the tyrosine of the collagen type II derived sequence Leu-Gln-Tyr-Met-Arg-Ala, is nitrated.

11. A method according to any of the claims 1 to 2 or 4 to 10 wherein said detection/ monitoring is carried out utilizing an antibody, which specifically recognizes a nitrated aromatic amino acid residue independent of the surrounding sequence and a second antibody, which recognizes a specific amino acid sequence located within the same protein, peptide or a fragment thereof as the nitrated amino acid residue.

12. A method according to any of the claims 1 or 3 to 10, wherein said detection/ monitoring is carried out utilizing an antibody, which specifically recognizes at least one nitrated aromatic amino acid residue in conjunction with a specific amino acid sequence.

13. An antibody raised against a peptide with the sequence $(X_{aa})_m$-His-Arg-Gly-Tyr:NO_2-Pro-Gly-Leu-Asp-Gly-$(X_{aa})_n$, wherein X_{aa} denote any amino acid or derivates thereof and m and n are independent integers from 0 to 10.

14. An antibody according to claim 13, in which said antibody is a monoclonal antibody.

15. A cell line producing the monoclonal antibody according to claim 14.

16. An antibody raised against a peptide with the sequence $(X_{aa})_m$-Leu-Gln-Tyr:NO_2-Met-Arg-Ala-$(X_{aa})_n$, wherein X_{aa} denote any amino acid or derivates thereof and m and n are independent integers from 0 to 10.

17. An antibody according to claim 16, in which said antibody is a monoclonal antibody.

18. A cell line producing the monoclonal antibody according to claim 17.

19. A method for monitoring a pathological process involving oxidative damage comprising the detection of a nitrated protein, peptide or fragment thereof, as defined in claim 1, versus the equivalent non-nitrated protein, peptide or fragment thereof.

20. A method according to claim 19, wherein the oxidative damage is associated with an inflammatory joint disease, comprising the detection of a cartilage matrix derived protein or fragment thereof containing at least one nitrated aromatic amino acid residue versus an equivalent protein or fragment without the nitrated aromatic amino acid residue(s).

21. A method according to any of the claims 19 to 20, comprising the steps of:

a) contacting an antibody, which specifically binds a nitrated epitope comprising at least one nitrated aromatic amino acid residue in conjunction with a specific amino acid sequence, with a biological sample.

b) contacting a second antibody which specifically binds the equivalent non-nitrated amino acid sequence as the antibody in a) with the same or an extract of the same biological sample;

c) determining a ratio between nitrated and non-nitrated peptide and/ or protein in said biological sample; and

d) evaluating ratios from a patient, in relation to ratios from healthy individuals.

22. A method according to claim 21, wherein an antibody as defined in any of the claims 15, 16, 18 or 19 is applied in step a).

23 A method according to any of the claims 19 to 22, wherein said detection provides means for an in-vitro diagnosis and/ or assessment of the severity of an oxidative damage and/ or inflammatory condition.

24. A method according to any of the claims 19 to 23, wherein the pathological process relates to cancer, Alzheimer's disease, Parkinson's disease, an inflammatory bowel disease, systemic lupus erythematosus, osteoarthritis or rheumatoid arthritis.

25. A kit for the use in detecting and/ or monitoring oxidative damage and/ or an inflammatory condition, comprising:

a) an antibody which specifically binds a nitrated epitope, comprising at least one nitrated aromatic amino acid residue in conjunction with a specific amino acid sequence; and

b) means for detection.

26. A kit according to claim 25, wherein a second antibody with specificity to the equivalent non-nitrated sequence is included.

27. A kit according to any of the claims 25 to 26, wherein the first antibody (a) is an antibody as defined in any of the claims 15, 16, 18 or 19.

28. A kit according to any of the claims 25 to 27, wherein a peptide between 6 and 20 amino acids, in which a succession of amino acids is equivalent to the binding epitope for one of said antibodies, is included.

Abstract

The present invention relates to a methods for improving the diagnostic possibilities of diseases where oxidative NO-modifications occur, for example inflammatory conditions, cancer, Parkinson's or Alzheimer's disease, and to provide means of monitoring the effects of therapeutical measures taken towards such diseases. The invention enable the detection of disease specific catabolic markers related to oxidative NO-modifications, utilizing an immunoassay comprising antibodies directed against nitrated and non-nitrated epitopes.

Figure 1

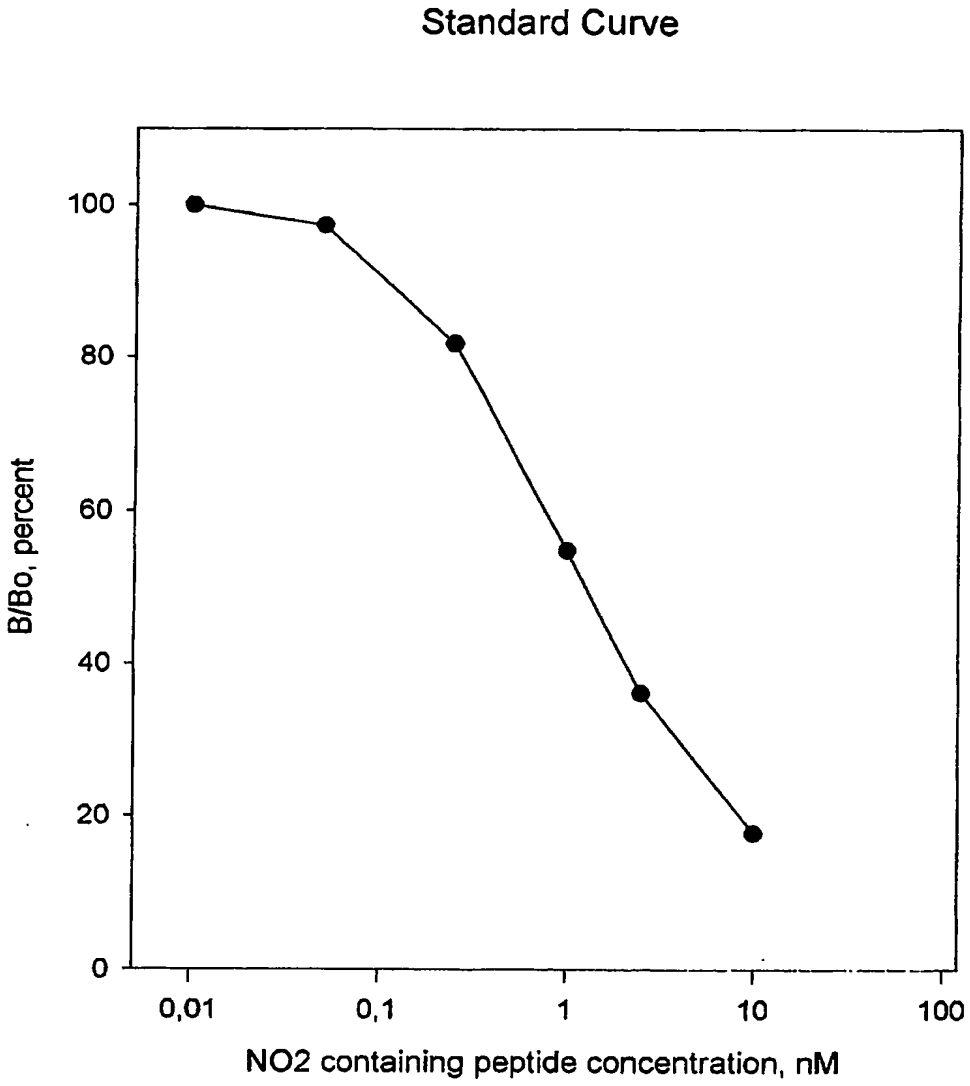

Figure 2